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Carcinogen-Induced DNA Amplification In Vitro: Overreplication of the Simian Virus 40 Origin Region in Extracts from Carcinogen-Treated CO60 Cells

YEHUDIT BERKO-FLINT,[1] SHULAMIT KARBY,[1] DAVID HASSIN,[1,2] AND SARA LAVI[1]*

Department of Microbiology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv,[1] and Department of Internal Medicine, Sheba Medical Center, Tel Aviv,[2] Israel

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An in vitro system to study carcinogen-induced amplification in simian virus 40 (SV40)-transformed Chinese hamster (CO60) cells is described. SV40 amplification in this system resembled in many aspects the viral overreplication observed in drug-treated CO60 cells. Cytosolic extracts from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated cells supported de novo DNA synthesis in the presence of excess exogenous T antigen and the SV40-containing plasmid $pSVK_1$. The pattern of viral replication in these extracts was unique, since only the 2.4-kilobase-pair region spanning the origin was overreplicated, whereas distal sequences were not replicated significantly. Extracts from control cells supported only marginal levels of replication. In HeLa extracts, complete SV40 DNA molecules were replicated efficiently. The overreplication of the origin region in CO60 cell extracts was bidirectional and symmetrical. A fraction of the newly synthesized DNA molecules underwent a second round of replication, yielding *Mbo*I-sensitive fragments representing the 2.4-kilobase-pair region around the origin. The mechanisms controlling the amplification of the viral origin region, the nature of the cellular factors induced in the carcinogen-treated cells, and their putative association with general drug-induced SOS-like responses are discussed.

Environmental agents play an important role in the initiation of human cancer. Several alterations in cellular processes have been recorded in tumor cells. These include mutations, chromosomal aberrations and rearrangements, gene amplification, DNA hypomethylation, and altered levels of gene expression (reviewed in references 3, 5, and 19). Earlier studies in our laboratory and by other groups demonstrated that DNA-damaging agents induce the amplification of viral and cellular genes (1, 21, 24–26, 37, 44). Enhancing gene amplification in this manner facilitates the analysis of the initial stages of this process.

A model system consisting of integrated simian virus 40 (SV40) sequences in virally transformed semipermissive Chinese hamster cells was used in our studies. After exposure to physical and chemical carcinogens and to agents arresting DNA replication, viral DNA synthesis is induced in these cells (24). The overreplication process is transient, reaching a maximal level 3 to 4 days after treatment. An active origin of replication and a functional T antigen were shown to be required for this phenomenon (25). The amplified DNA is heterogeneous in size and is enriched for sequences spanning the 2-kilobase-pair (kb) region around the origin of DNA replication (26). SV40 overreplication is bidirectional (26) and is aphidicolin sensitive (14, 20), suggesting the involvement of the cellular DNA replication machinery in this process. Similar activation of polyomavirus in drug-treated virally transformed rat cells was reported by several groups (11, 23, 29, 34). Cell fusion experiments demonstrated that the induction of SV40 and polyomavirus DNA synthesis is mediated by carcinogen-induced *trans*-acting factors (4, 23, 31) that are transmitted upon fusion from the treated cells to the untreated cells harboring the integrated viral genome.

Recently, we have shown that *trans*-acting factors are involved in another carcinogen-induced process: enhanced gene expression (20, 22). It was previously demonstrated that exposure of various cell lines to environmental agents results in elevated expression of several viral and cellular genes (15, 17, 20, 22, 35, 45). This process is regulated by cellular factors, is not dependent on DNA replication, and is controlled at the levels of transcription and posttranscription (20, 22; T. Kleinberger, manuscript in preparation). The nature of the factors involved in the enhanced expression and their possible role in the overreplication phenomenon is not yet known.

To facilitate analysis of the *trans*-acting factors responsible for viral overreplication, an in vitro system for DNA amplification was established in our laboratory (4). This system is based on the in vitro system for SV40 replication developed recently by Li and Kelly (27). By using the in vitro system for SV40 replication, it was demonstrated by several laboratories that cytosolic extracts from permissive cells supplemented with purified T antigen efficiently replicate exogenous SV40 DNA templates containing a functional origin of replication, yielding complete closed circular DNA molecules (16, 18, 27, 43, 49). Cytosolic extracts from Chinese hamster and other nonpermissive cell lines failed to support significant viral DNA replication (28).

The in vitro amplification system described here consists of cytosolic extracts from drug-treated SV40-transformed Chinese hamster (CO60) cells (24), template SV40 DNA molecules, and exogenous T antigen. This system provides an excellent tool for analysis of the mode of DNA amplification, characterization of the amplified DNA, and purification of the cellular factors responsible for the amplification process. The availability of this system will facilitate studies on the molecular mechanisms of gene amplification, on the control of cellular permissivity to viral replication, and on the putative existence of carcinogen-induced SOS-like responses.

* Corresponding author.

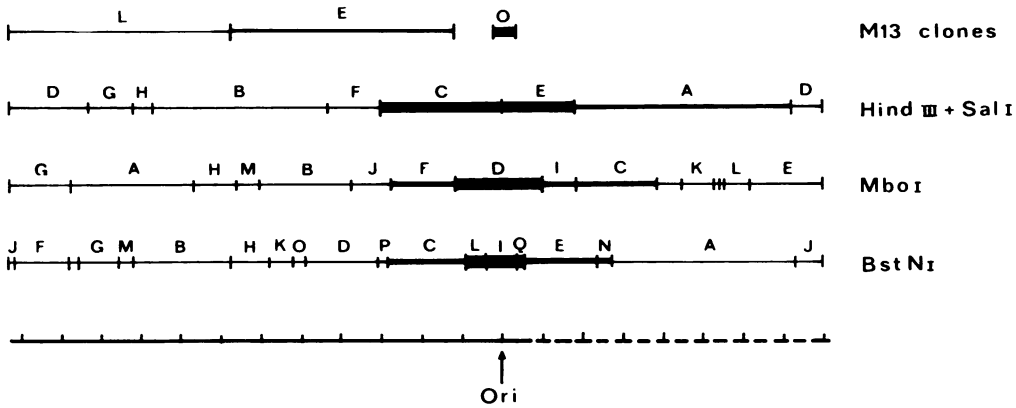

FIG. 1. Restriction maps of plasmid $pSVK_1$. The bottom line represents the distribution of the SV40 (———) and pML2 (– – – –) sequences within the 8.24-kb plasmid $pSVK_1$. The distance between the tick marks is 400 bases. The SV40 origin (Ori) is marked. In the restriction maps of the plasmid cleaved by *Bst*NI, *Mbo*I, and *Hin*dIII plus *Sal*I, the letters are according to fragment size. In the diagram showing the position of the SV40 fragment cloned into M13, L represents late region (*Kpn*I-*Bam*HI), E represents early region (*Taq*I-*Bam*HI), and O represents origin (*Hin*dIII-*Sph*I). Bars represent regions that underwent overreplication to the highest extent; thick lines represent regions that were replicated to a lower degree. Amplification of the *Bst*NI and the *Mbo*I fragments was determined by restriction enzyme analysis (Fig. 4 and 7, respectively); amplification detected by the *Hin*dIII-*Sal*I fragments and M13 clones was deduced from the hybridization results (Fig. 5 and 6, respectively).

MATERIALS AND METHODS

Cells. HeLa, CO60 (24), 814 (20), and BSC-1 cells were propagated in monolayer cultures in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Biolabs, Jerusalem, Israel).

Plasmids and bacteriophages. $pSVK_1$ (8.24 kb), a plasmid containing the entire SV40 genome cloned into pML2 (a derivative of pBR322), was kindly provided by D. Dorsett (10). pNIS80, an origin-defective plasmid harboring a deletion in the *Bgl*I site, was kindly provided by A. Sergeant (40). Phages mp18SVEC and mp19SVENC contain the 2-kb-long coding and noncoding early strands, respectively, of SV40 (*Taq*I-*Bam*HI) cloned in the M13 phage vectors mp18 and mp19; mp18SVLNC and mp19SVLC contain the 2-kb noncoding and coding strands, respectively, from the SV40 late region (*Kpn*I-*Bam*HI). M13 Ori1 and M13 Ori2 are phages containing the 0.2-kb *Hin*dIII-*Sph*I origin fragments. These phages were kindly provided by B. Stillman (32).

Preparation of cell extracts. Crude cytosolic extracts were prepared from monolayer cultures as described by Li and Kelly (27). CO60, 814, and HeLa cells (5×10^6 each) were seeded onto 14-cm-diameter dishes. At 24 h after seeding, CO60 and 814 cells were treated for 2 h with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Aldrich Chemical Co., Inc., Milwaukee, Wis.) freshly dissolved in dimethyl sulfoxide. At the end of the treatment, the medium was replaced in both treated and control cultures. At 24 h after treatment, cytosolic extracts were prepared from control and treated cells. HeLa cell extracts were prepared 24 h after seeding. Five dishes of cells routinely yielded 1.5 ml of extract (3.5 to 7.0 mg of protein per ml).

T-antigen purification. T antigen was purified as described by Dixon and Nathans (9). Monolayer cultures of BSC-1 cells were grown in 14-cm-diameter dishes, and 24 h after seeding the cells were infected (multiplicity of infection of 20) with the SV40 mutant CS1085 (8). At 48 h after infection, T antigen was purified by immunoaffinity chromatography on a column containing monoclonal anti-T-antigen antibody PAb419 (13).

Replication reaction. Standard 50-μl reaction mixtures contained (final concentrations) 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM each dATP, dGTP, and dTTP, 25 μM [α-^{32}P]dCTP (specific activity, 1×10^3 to 3×10^3 cpm/pmol; The Radiochemical Center, Amersham, England), 4 mM ATP, 200 μM each CTP, GTP, and UTP, 40 mM creatine phosphate, 1 μg of creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.), 0.20 μg of supercoiled template DNA ($pSVK_1$ or pNIS80), 180 μg of cytosolic extract, and purified T antigen as specified. The reaction mixtures were incubated at 37°C for 3 to 4 h unless otherwise stated and then terminated by the addition of an equal volume of stop buffer containing 0.4% sodium dodecyl sulfate and 20 mM EDTA.

Analysis of replication products. To measure the extent of DNA synthesis, samples were removed and added to 50 μg of denatured, sheared salmon sperm DNA as carrier and then mixed with 0.5 ml of 10% trichloroacetic acid (TCA) in 1% sodium pyrophosphate for 20 min on ice. The precipitates were collected by filtration onto GF-C glass fiber filters (Whatman, Inc., Clifton, N.J.), followed by three washes with ice-cold 10% TCA and two washes with ethanol. The filters were then counted by liquid scintillation counter.

To purify the DNA, the replication reaction mixtures were digested by proteinase K (200 μg/ml, 60 min, 37°C; Merck & Co., Inc., Rahway, N.J.) and then extracted once with phenol and once with chloroform. The DNA was separated from unincorporated nucleoside triphosphates on a Sephadex G-50 column (Pharmacia, Inc., Piscataway, N.J.), extracted once with chloroform, and ethanol precipitated.

Restriction enzyme digestions were carried out in 30-μl reaction mixtures under conditions specified by the manufacturer (New England BioLabs, Inc., Beverly, Mass.). For further analysis, samples of the replication reactions were hybridized to blots containing $pSVK_1$ DNA digested by *Hin*dIII and *Sal*I (Fig. 1) or to dot blots containing 1.0 μg of the different M13 constructs harboring the indicated single-stranded regions of the SV40 genome (Fig. 1). Hybridization was performed as described elsewhere (21).

RESULTS

Kinetics of SV40 replication in cell extracts from treated CO60 cells. To characterize the mode of SV40 amplification

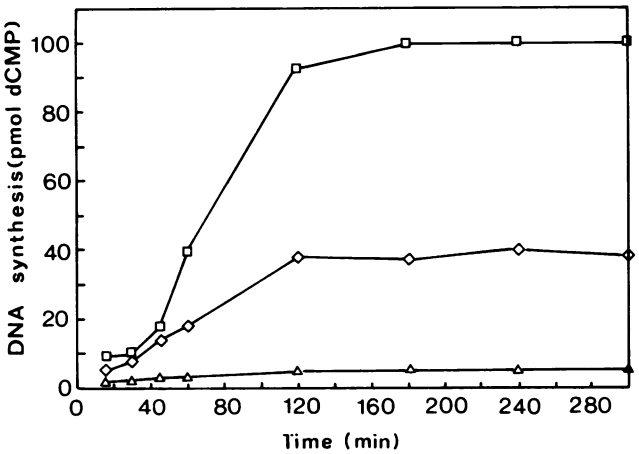

FIG. 2. Kinetics of in vitro SV40 DNA synthesis in reactions catalyzed by extracts from HeLa cells and from MNNG-treated and untreated CO60 cells. Extracts were prepared from exponentially growing HeLa cells (□) and from nontreated (△) and treated (7 μg of MNNG per ml for 2 h; ◇) CO60 cells 24 h after treatment as described in Materials and Methods. Standard 50-μl reaction mixtures were as described in Materials and Methods. Reactions were carried out in the presence of 0.6 μg of T antigen. The reaction mixtures were incubated at 37°C. At the indicated times, 5-μl samples were removed to measure TCA-precipitable counts. The incorporation of dCMP into DNA was determined after substraction of dCMP incorporation in parallel reactions lacking T antigen.

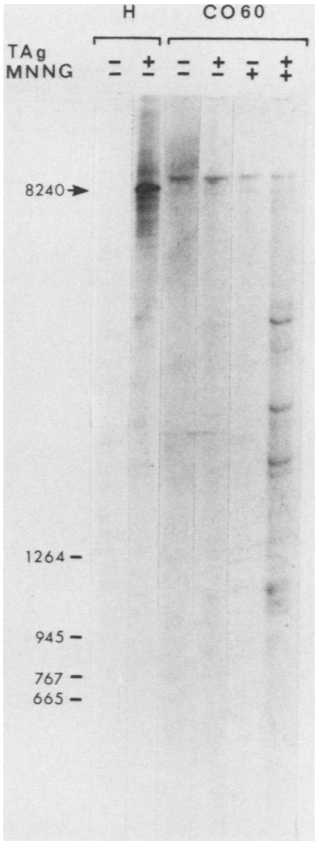

FIG. 3. *Dpn*I analysis of replication products. In vitro replication reactions were performed as described in the legend to Fig. 2 in the absence or presence of T antigen (TAg; 0.6 μg). The reaction mixtures were incubated at 37°C for 3 h and then terminated by the addition of 10 mM EDTA and 0.2% sodium dodecyl sulfate. The DNA was processed as described in Materials and Methods, followed by *Dpn*I-*Sal*I digestion. The digestion products were separated by electrophoresis on 1.0% agarose gels, which were then dried and exposed for autoradiography. H, HeLa. The arrow indicates the position of full-length (8,240 bp) $pSVK_1$. Other numbers represent the positions and sizes (in base pairs) of the largest *Dpn*I digestion products of $pSVK_1$.

in virally transformed CO60 cells and to analyze the cellular factors controlling this process, the in vitro system for SV40 replication developed by Li and Kelly (27) was used. With this approach, viral DNA synthesis catalyzed by extracts prepared from drug-treated CO60 cells was studied.

Twenty-four hours after MNNG treatment, crude cytosolic extracts were prepared from treated and untreated CO60 cells and from HeLa cells. The reaction mixtures included cytosolic extracts, supercoiled $pSVK_1$ DNA, and purified T antigen. Similar reactions were carried out in the absence of T antigen. Samples were removed at different time intervals, and the level of DNA synthesis was measured by TCA precipitation. The T-antigen-dependent incorporation of dCMP into DNA was determined (Fig. 2). SV40 replication in extracts from control untreated CO60 cells was inefficient, yielding only 3 to 5 pmol of incorporated dCMP after 3 h of incubation. These results are in agreement with previous reports on SV40 replication in extracts from Chinese hamster cells (28). However, in extracts from the treated CO60 cells, incorporation of dCMP was initiated immediately, and the typical lag period occurring in reactions catalyzed by HeLa extract was not detected (43). In the reaction directed by treated CO60 extract, 40 pmol of dCMP was incorporated after 3 h of incubation. Thus, treatment of the nonpermissive CO60 cells with MNNG yielded cytosolic extracts in which enhanced T-antigen-dependent SV40 replication occurred. The reaction catalyzed by the permissive HeLa cells extract was more efficient, and 100 pmol of dCMP was incorporated into DNA.

Complete SV40 DNA molecules are not synthesized in reactions catalyzed by extracts from MNNG-treated CO60 cells. To analyze the replication products, the purified DNA was digested by *Dpn*I and *Sal*I. The template plasmid $pSVK_1$, which was used in all experiments, was isolated from *Escherichia coli* (*dam*$^+$) and was fully methylated. It was therefore sensitive to *Dpn*I, which cleaves methylated DNA on both strands, yielding multiple fragments (the largest of which is 1,264 base pairs [bp]). Newly synthesized, unmethylated DNA is resistant to the enzyme (28). The DNA synthesized by the HeLa extract in the presence of T antigen was resistant to *Dpn*I, and most of the radioactivity migrated as full-length linear DNA molecules after cleavage with *Sal*I, which linearizes $pSVK_1$ DNA (Fig. 3). The DNA molecules produced by the treated CO60 extracts in the presence of T antigen displayed a different pattern. Most of the *Dpn*I-resistant newly synthesized DNA molecules migrated as a heterogeneous collection of DNA fragments. In addition, several distinct bands of 1.7 to 3.4 kb were detected. The nature of these discrete fragments, which are larger than the *Dpn*I-sensitive digestion products of $pSVK_1$, is under investigation. Thus, most of the DNA molecules synthesized in the reaction catalyzed by extracts from the drug-treated CO60 cells resulted from incomplete replication of the input plasmid. Such replication products were not produced in reactions directed by extracts from untreated control cells. A very faint band of newly synthesized, full-length, DpnI-resistant molecules was detected in reac-

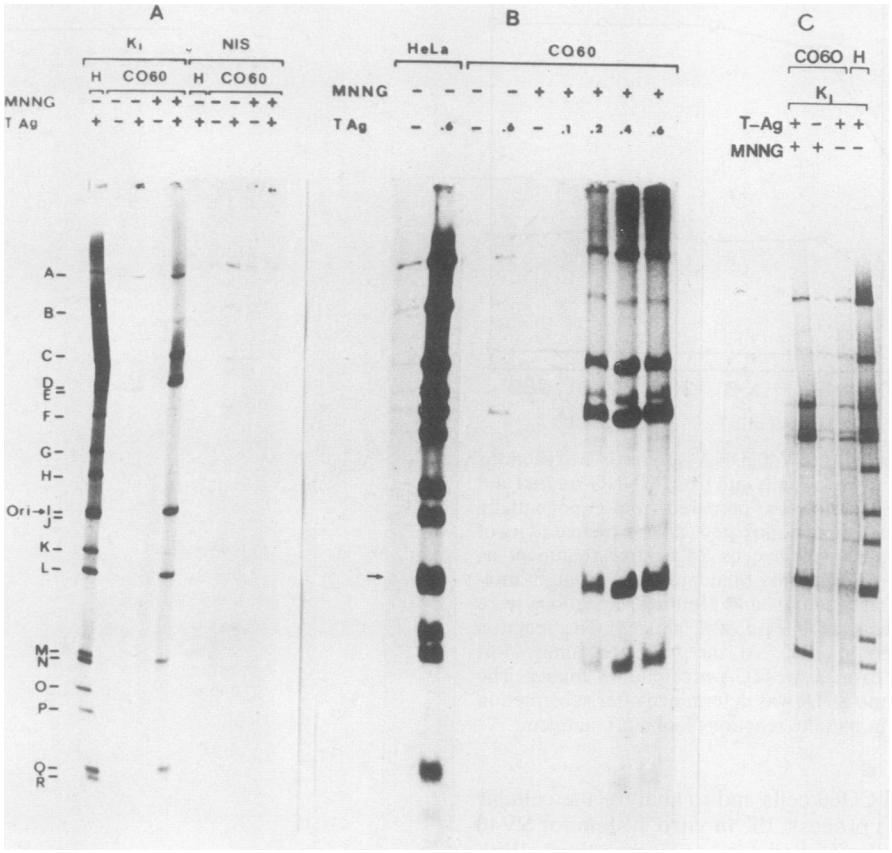

FIG. 4. Overreplication of origin fragments as demonstrated by *Bst*NI restriction analysis. The in vitro replication reaction mixtures were incubated for 3 h at 37°C as described in the legend to Fig. 2. The replication products were purified, digested with *Bst*NI for 6 h at 60°C under paraffin oil, electrophoresed through 6% polyacrylamide gels, dried, and subjected to autoradiography. The detectable fragments are listed according to size (fragment A [1,860 bp] to fragment R [55 bp]). The origin (Ori)-containing fragment (I) is marked. (A) The template DNA used was either $pSVK_1$ or the SV40 origin-deleted plasmid pNIS80 (40). T antigen (TAg; 0.6 μg) was added where indicated. In the reaction directed by the HeLa extract (H), the radioactivity incorporated into fragments A and B was slightly reduced as a result of nucleolytic activity. In panels B and C, $pSVK_1$ was used as a substrate, and T antigen (0.6 μg) was added as indicated.

tions catalyzed by control extracts in the presence of exogenous T antigen and by treated CO60 extracts in the presence or absence of T antigen. The synthesis of this minor population of molecules was not enhanced when extracts from treated cells were used. The band containing molecules larger than full-length $pSVK_1$ represents endogenous cellular DNA present in the CO60 extracts, since it appeared also in the absence of exogenous template when extracts from control and treated cells were used (data not shown).

The replication products obtained in the reactions catalyzed by the treated CO60 extracts differed from those produced by the HeLa extracts. It was therefore important to establish that the atypical synthesis was dependent on a functional origin of replication and T antigen. Moreover, identification of the nature and the structure of these molecules was required.

T-antigen and origin-dependent overreplication of the 2.3-kb region surrounding the origin. To characterize the replication products from the reaction directed by extracts from drug-treated CO60 cells, the DNA was digested with *Bst*NI. This enzyme has multiple recognition sites on the $pSVK_1$ genome, yielding 18 detectable fragments upon electrophoresis through a 6% polyacrylamide gel (Fig. 4). The radioactive digestion products obtained from the HeLa-derived reactions in the presence of T antigen displayed all 18 fragments, which were labeled according to size. However, the newly replicated fragments synthesized by the treated CO60 extracts displayed a very specific replication pattern that was not affected even upon addition of increasing concentrations of T antigen (Fig. 4). Only a subset of the fragments representing the 2.3-kb region spanning the origin of DNA replication appeared (fragments C, E, I, L, N, and Q; Fig. 1 and 4). The T-antigen-dependent overreplication of the viral origin sequences is evident upon comparison of the synthesis of fragments I and J, of 311 and 307 bp, respectively. In the reactions catalyzed by the HeLa extracts, the two fragments were replicated to the same extent (Fig. 4), whereas in the reactions directed by the treated CO60 extracts, only fragment I, containing the origin of DNA replication, was replicated; fragment J, residing about 180° from the origin, was not synthesized.

The overreplication of SV40 in extracts from treated CO60 cells was origin dependent. When pNIS80, a plasmid harboring a deletion within the core origin, was used as a template, no replication was observed in reactions catalyzed by CO60 or HeLa extracts in the presence of excess T antigen.

Analysis of the pattern of viral DNA replication strongly suggested that in extracts from treated CO60 cells, replication proceeded bidirectionally from the viral origin of DNA

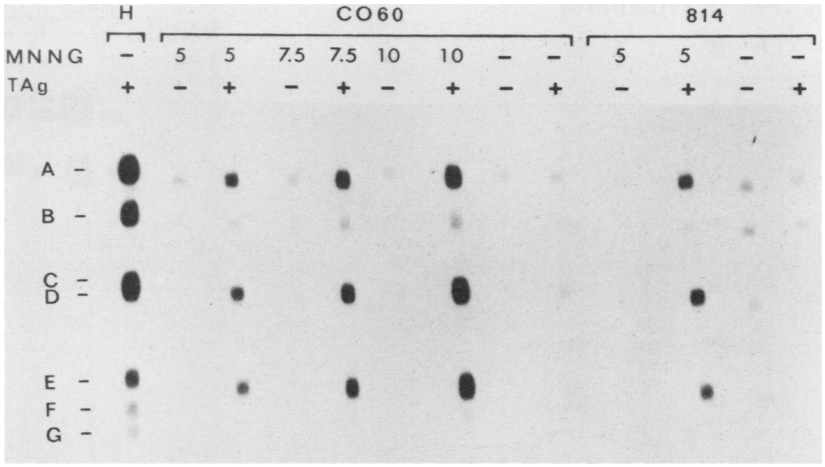

FIG. 5. Demonstration that the level of overreplication is proportional to the extent of DNA damage. $pSVK_1$ DNA (30 μg) was digested by *Hin*dIII plus *Sal*I and loaded on a wide slot across a 1.2% agarose gel. After electrophoresis, the DNA was blotted onto nitrocellulose filters, and 3-mm-thick strips were hybridized against replication products from reactions catalyzed by HeLa extract (H) and MNNG-treated (5 to 10 μg/ml) and untreated CO60 and 814 cells. The reactions were carried out with $pSVK_1$ as a template and 0.6 μg of T antigen (TAg).

replication. Similar levels of replication were observed for fragments C and E, flanking both sides of the origin. Fragment C carries viral sequences, whereas fragment E is of pBR origin (Fig. 1 and 4). A similar pattern of overreplication in the vicinity of the origin was observed when several other wild-type SV40 constructs of similar size were used (data not shown). In extracts from control cells, no SV40 replication was observed even in the presence of excess T antigen (Fig. 4B), indicating that in addition to T antigen, cellular factors induced by the carcinogens are required for the amplification.

Enhanced DNA damage increases the level of origin overreplication. Extracts were prepared from CO60 cells exposed to increasing concentrations (5 to 10 μg/ml) of MNNG. The replication products were hybridized to blots containing $pSVK_1$ DNA digested by *Hin*dIII and *Sal*I (Fig. 1 and 5). The HeLa replication products hybridized to fragments A to G (hybridization to fragment H was below the detection level). The extent of hybridization was proportional to fragment size. A different pattern of hybridization was displayed by the treated CO60 replication products. Of the $pSVK_1$ fragments, only fragments A, C, and E, which are located in the origin region, hybridized. (Fragments C and D are not well resolved on the blot, and further analysis revealed that fragment C was the one that was overreplicated [data not shown].) Fragment A, which is 2.3 kb long, is located 700 bp from the origin; hybridization to this fragment resulted from the amplification of a portion of this fragment, i.e., the region adjacent to fragment E. Fragment B, which is located 1.8 kb away from the origin, displayed a very low level of hybridization. The extent of hybridization to the origin fragments (C and E) was proportional to the level of DNA damage, suggesting that the activity of the cellular amplification factors induced in the treated cells was directly related to the extent of damage. In control reactions, a very low level of hybridization to origin sequences was observed. All of the replication reactions were T antigen dependent; very low hybridization levels were observed in reactions conducted in the absence of T antigen.

MNNG-treated extracts derived from another SV40-transformed Chinese hamster cell line (814), harboring an origin-defective SV40 genome, yielded a similar pattern of origin overreplication (Fig. 5). Thus, the cellular factors responsible for the amplification were not restricted to CO60 cells but could be induced in other SV40-transformed cells.

Symmetrical overreplication of the origin region. To determine whether viral replication is symmetrical and both leading and lagging strands of the template $pSVK_1$ molecules are evenly replicated, single-stranded M13 clones containing different regions of the SV40 genome (Fig. 1) were immobilized on nitrocellulose filters and hybridized to the labeled replication products (Fig. 6, lanes A). The efficiency of hybridization of the DNA on the blots to evenly labeled DNA was determined by rehybridization of the blots (after the removal of the hybrids) to homogeneously labeled $pSVK_1$ DNA (Fig. 6, lanes B). The autoradiograms were scanned, and the levels of hybridization were calculated. The HeLa reaction products hybridized similarly to the homogeneously labeled $pSVK_1$ DNA (Fig. 6 and Table 1), demonstrating that the different regions of the template were symmetrically and evenly synthesized. It should be noted that the origin fragments (1 and 2) were 10% the size of the other fragments and therefore hybridized to a lower extent. The hybridization pattern of DNA synthesized by the treated CO60 cell extracts was different (Fig. 6). Despite the small size of the origin fragments, slots containing DNA from both origin strands displayed a pronounced hybridization much higher than that obtained by the HeLa replication products. Substantial hybridization was depicted by using both strands of the early region, which reside 0.5 kb from the origin (Fig. 1). The fragments representing the late region, which are remote from the origin, hybridized less efficiently. No hybridization was observed when products from the control extract were hybridized. The level of amplification of the sequences spanning the origin region (fragments 1, 2, 4, and 6) was compared with levels of the sequences distal to it (fragments 3 and 5). The two origin strands were amplified similarly (11.7- and 15.1-fold), whereas both strands from the adjacent fragment were less amplified (5.3- and 8.5-fold) (Table 1). These results demonstrate that the sequences within the origin region were overreplicated symmetrically.

Rereplication of the origin region. To examine the possibility that a second round of replication occurred in the in vitro replication reactions, the replication products were

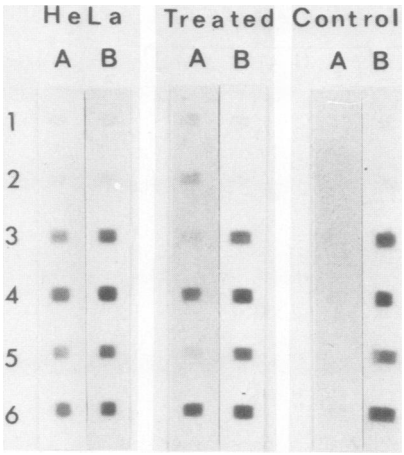

FIG. 6. Symmetrical overreplication of the origin region. In vitro overreplication reactions were carried out as described in the legend to Fig. 2. The labeled products from reactions catalyzed by extracts from HeLa cells and from treated and control CO60 cells were hybridized to the single-stranded M13 DNA clones on nitrocellulose filters. By using a slot blot apparatus, 1.0 μg of DNA from each clone was immobilized on slots 1 to 6. The blot was later cut into three identical strips, which were hybridized to each of the three different radioactive probes (lanes A). Slots: 1 and 2, M13 clones harboring the origin strands (*Hin*dIII-*Sph*I fragments; 200 bases); 4 and 6, clones with the plus and minus early-region strands (*Taq*-*Bam*HI fragments); 3 and 5, clones with the plus and minus late-region strands (*Kpn*I-*Bam*HI fragments). The early- and late-region fragments are 2,000 bases long. Positions of these fragments are shown in Fig. 1. After autoradiography, the hybridized DNA was removed and the filters were rehybridized to nick-translated $pSVK_1$ probe (lanes B) to determine the level of hybridization of the DNA on the slots to homogeneously labeled DNA.

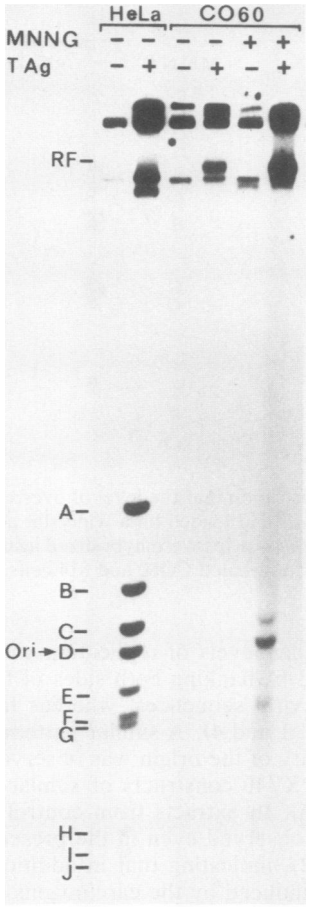

FIG. 7. Rereplication of the origin region. Rereplication products from the reactions described in the legend to Fig. 5, which were catalyzed by extracts from HeLa, control, and MNNG-treated (10 μg/ml) CO60 cells, were subjected to *Mbo*I digestion. The products were separated on a 1.0% agarose gel, which was then dried and exposed for autoradiography. TAg, T antigen; RF, replicating forms; Ori, origin region.

digested with *Mbo*I, an enzyme that cleaves DNA which is unmethylated on both strands. Since the substrate DNA is fully methylated, only molecules undergoing two rounds of replication will be digested by this enzyme. A fraction of the HeLa replication products was cleaved by this enzyme, yielding 10 detectable fragments (A to J) representing the evenly rereplicated $pSVK_1$ genome (Fig. 7). DNA molecules that were replicated only once or underwent partial replication migrated at the top part of the gel. The rereplication reaction occurred only in the presence of T antigen. The reaction products from control CO60 cells did not yield *Mbo*I-sensitive bands even in the presence of excess T antigen, whereas four *Mbo*I-sensitive fragments (C, D, F, and I) representing the 2.4-kb region around the origin were obtained from reactions catalyzed by extracts from MNNG-treated CO60 cells. The rereplication was origin dependent (data not shown). Most of the *Mbo*I-sensitive fragments were heterogeneous in size, migrating as background. The *Mbo*I-sensitive products from both HeLa and treated CO60 extracts comprised about 10% of the newly replicated DNA.

TABLE 1. Hybridization of single-stranded M13 DNA clones[a]

Fragment	Relative value							
	HeLa				CO60 treated			
	A	B	A/B	Amplification	A	B	A/B	Amplification
1	2.6	2.2	1.18	1.3	6.8	2.9	2.3	11.7
2	2.3	2.3	1.00	1.1	10.1	3.3	3.0	15.1
3	17.9	19.5	0.91	1.0	3.7	18.4	0.2	1.0
4	26.8	29.9	1.05	0.98	31.2	29.1	1.07	5.35
5	20.4	20.2	1.00	1.1	4.5	20.5	0.21	1.0
6	30	25.8	1.16	1.3	43.7	25.6	1.7	8.51

[a] The autoradiograms shown in Fig. 6 were scanned with a densitometer, and relative synthesis of the in vitro replication products (A) and relative hybridization of the homogeneously labeled DNA (B) were calculated. The specific replication of each fragment compared with its level in the homogeneously labeled DNA was calculated (A/B). The ratio between the specific replication of each fragment in the vicinity of the origin (1, 2, 4, and 6) and the specific replication of the distal fragments (3 and 5) represents the amplification level.

DISCUSSION

This paper has described an in vitro system to study SV40 amplification in extracts from carcinogen-treated SV40-transformed Chinese hamster cells. Exogenous T antigen, intact origin sequences, and carcinogen-activated cellular factors were shown to be essential for overreplication in vitro. Analysis of the replication products revealed that despite the elevated DNA synthesis in extracts from the treated cells, the production of full-length *Dpn*I-resistant molecules was not enhanced (Fig. 3). Detailed analysis of the

replicating DNA molecules demonstrated that the 2.4-kb region spanning the origin was overreplicated. Amplification of the origin region was bidirectional (Fig. 4 and 5), symmetrical (Fig. 6), and sensitive to aphidicolin (Y. Berko, unpublished data), and a small portion of the molecules underwent two rounds of replication (Fig. 7). In earlier in vivo experiments, after treatment of CO60 cells with carcinogens, we observed bidirectional amplification of the 2-kb region around the origin. This replication was sensitive to aphidicolin. Thus, the SV40 amplification in treated cells could be duplicated in vitro when extracts from the treated CO60 cells were used.

SV40-transformed CO60 cells express T antigen, and the level of this protein is elevated in carcinogen-treated cells (20, 22). This endogenous T antigen is not sufficient to support viral replication in extracts from treated cells, and the reactions are dependent on exogenous T antigen. However, the internal T antigen contributes to the ability of the extracts to support viral replication. Extracts from control or treated Chinese hamster cells (CHO or CHE) that do not harbor viral genetic information did not facilitate SV40 replication in vitro even in the presence of exogenous T antigen (A. Parket, manuscript in preparation), whereas extracts from treated CO60, 814 (Fig. 5), and other SV40-transformed cell lines did. The possible role of the endogenous T antigen is discussed below.

The studies presented here demonstrate that T antigen is not the only factor required for viral replication in these cells. In reactions catalyzed by extracts from untreated CO60 cells, the addition of excess exogenous T antigen did not facilitate SV40 replication. T-antigen-dependent viral replication occurred only in extracts from treated CO60 cells. These findings suggest that cellular factors controlling the amplification process are activated in the treated cells. These factors can be directly activated as a result of cellular processes induced by the carcinogen (20, 22) or as a result of their activation by the endogenous T antigen, which is known to be a *trans*-acting protein (2, 38, 39). The level of the carcinogen-induced factors is positively related to the extent of DNA damage, since exposure of CO60 cells to increasing levels of MNNG enhanced the amplification potential of the extracts (Fig. 5). The cellular factors controlling amplification can be of positive or negative nature and can function either independently or in concert with the endogenous T antigen. Studies in several laboratories have demonstrated that T antigen indeed is capable of interacting with cellular proteins and thus of effecting viral replication. T antigen was shown to be associated with polymerase-primase complex and p53 (6, 12, 41). Moreover, species specificity of these proteins is an important determinant in the tropism for SV40 replication in vivo and in vitro (6, 30, 47).

Our data suggest that the inability of Chinese hamster cells to provide permissive conditions for viral replication stems from deficiencies in both the initiation and the elongation phases. In treated cells, the barrier to the initiation of viral DNA replication is removed. Analysis of the kinetics of DNA replication in treated CO60 cell extracts suggests that the presynthesis reaction in these cells is efficient. The lag period observed in HeLa extracts before viral DNA synthesis (43) is shortened in reactions directed by treated CO60 extracts (Fig. 2).

Studies by several groups have indicated that during the presynthesis stage, an unwinding reaction occurs that is effected by T antigen (50) and by cellular proteins, including RP-A and RP-C (46). Alterations in the activities of these proteins might have occurred in the treated cells. Earlier studies in our laboratory (Y. Berko, Ph.D. thesis, Weizmann Institute of Science, Rehovot, Israel, 1988) and by others (17, 36) have demonstrated that a prolonged S phase occurs after treatment with carcinogens. Cellular factors associated with the S phase might be responsible for the amplification (7). It is possible that the factors controlling the enhanced initiation in extracts from S-phase HeLa cells (33) are those induced in the carcinogen-treated CO60 cells. However, cellular proteins associated with other stages in the initiation of viral DNA replication might be influenced. The nature of the cellular factors and their function is currently being investigated.

Restriction enzyme analysis of the replication products demonstrated that the 2.4-kb region around the origin of viral replication in the treated cells extracts was overreplicated. Recent electron microscopy studies (S. Bratusin, manuscript in preparation) revealed that the arms of most theta forms are 0.3 to 2.6 kb in size, demonstrating that the replication forks are prematurely arrested. A similar pattern of origin overreplication was demonstrated when other SV40 plasmids of similar size, containing different viral or plasmid sequences in the vicinity of the origin, were used. These data suggest that the arrest of DNA replication did not result from termination signals on the template DNA. Thus, cellular factors involved in DNA elongation might be inefficient in Chinese hamster cells.

Most of the replication products underwent incomplete synthesis, and less than 1% of the *Dpn*I-resistant newly synthesized products were full-length molecules. However, a fraction (5 to 10%) of the newly synthesized DNA was rereplicated, yielding *Mbo*I-sensitive fragments representing the 2.4-kb region around the origin. It is possible that these fragments arose from multiple reinitiation events occurring on partially replicated duplexes, giving rise to onion skin complexes. Alternatively, arrest of the replication forks can lead to aberrant types of replication and recombination, yielding hairpin structures and various duplications, such as direct and inverted repeats that are commonly found in amplified DNA (36, 42). Detailed analysis of the structure of the amplified DNA will contribute to an understanding of the mechanism(s) governing DNA amplification at arrested replication forks.

Our studies suggest that a unique set of cellular responses is activated as a result of DNA damage. These responses might control both the enhanced gene expression described earlier by our group and the enhanced amplification described here. The carcinogen-induced responses might be analogous to the SOS system documented in bacteria (48). The in vitro amplification system might facilitate analysis and identification of the cellular factors involved in these responses.

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